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Breast Cancer Patients Whose Tumors Overexpress HER-2/neu

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13. ABSTRACT (Maximum 200 Words)

The goal of this research is to develop stable radioimmunoconjugates (RICs) of *anti-HER2/neu* antibodies (MoAbs) for imaging and therapy in an animal model bearing human breast tumors. Pharmacy-grade Herceptin and an *anti-HER2/neu* antibody were conjugated with a DTPA linker. $^{111}InCl_3$ and $^{90}YCl_3$ were labeled to MoAb-CHX-A''–DTPA according to the procedure of Clarke et al. (*Cancer Res* 60: 4804, 2000). Binding capacity of RICs was tested with human cancer cell lines MCF-7, HCC-1954, BT-474 and SKBR-3. Results: The molar ratio of DTPA-CHX-A'' to MoAbs was 1.4:1. Using a molar excess of 10:1 CHX-A''-DTPA to MoAb, a specific activity of 1.87 µCi ^{111}In/µg MoAb-RIC and 2.71 µCi ^{90}Y/µg MoAb-RIC was obtained. The purity of RIC was 96%+ for ^{111}In and 99%+ for ^{90}Y. Stability at 37° C for both RICs ranged from 98% at 24 hours in human plasma to 85% at 96 hours. ^{111}In- RIC bound to MCF-7 cells with a binding radio of 2.5:1 using a nonspecific IgG RIC as a control. A 98.3:1 binding ratio was observed when SKBR-3 cells were exposed. Conclusion: MoAbs can be labeled with ^{111}In and ^{90}Y using a CHX-A''-DTPA linker. Stable RICs are formed and significant cell binding of RICs occurs when *HER2/neu* over-expressing cells are used.

14. SUBJECT TERMS
Breast Cancer, Radioimmunotherapy, Radioimmunodetection, HER2/neu

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INTRODUCTION

Many women continue to present to their physician with locally advanced or metastatic breast cancer. A portion of these patients may have a particularly aggressive form of this disease with little or no clinical signs of the disease. Biopsy evaluation as well as the measurement of serum tumor markers, hormone receptor status, and certain epidermal growth receptor proteins on the surface of tumor cells all help to determine the grade and aggressiveness of a particular cancer. Between 25 and 30% of all breast cancer patients have an aggressive form of breast cancer known as *HER2/neu* positive disease. The presence of *HER2/neu* over-expression signifies a poor prognosis for these patients. Innovative techniques and treatment modalities are needed to improve the outcomes of these particular breast cancer patients.

The long-term goal of this investigation is to determine if adding a diagnostic isotope known as Indium-111 (^{111}In) and alternately, a therapeutic isotope know as Yttrium-90 (^{90}Y) to Herceptin® can improve its effectiveness as an anticancer agent. Adding ^{111}In to Herceptin® may convert this monoclonal antibody (MoAb) into an effective diagnostic staging tool. Adding ^{90}Y to Herceptin® may improve its cytotoxic efficacy without adding significant risk to the patient. The purpose of this second year-end report is to describe the results of studies completed in our investigations to date. The first year report (Exhibit B) described the labeling of Herceptin®, *anti-HER2/neu*, and nonspecific IgG antibodies with ^{111}In and ^{90}Y using both DOTA and CHX-A" DTPA linkers. Since that report a standard procedure for making these radioimmunoconjugates (RICs) has been established in our laboratory. New RICs are being produced and tested for chemical and <u>in vitro</u> stability according to these standard procedures. Biodistribution and imaging studies were performed and completed. Therapy trials are currently underway. Results of completed studies are presented in the order in which they were proposed in the original approved grant.

BODY

OBJECTIVE 1: To synthesize ^{111}In- and ^{90}Y-*anti-HER2/neu* antibody conjugates and perform <u>in vitro</u> analyses of stability, specificity, and affinity for normal expressing and over expressing HER2 human breast cancer cells.

The investigations described for **Objective 1** have been completed and the results of this portion of the study are being published (<u>Cancer Biotherapy and Radiopharmaceuticals</u>, 2003, in press). A copy of this manuscript proofs and several abstracts are attached to this report.

OBJECTIVE 2: Construction of a recombinant bicistronic onco-retrovirus for the stable genetic transfer of the *HER2/neu* cDNA and a fluorescent marker protein cDNA (enYFP) in tandem into breast cancer cell lines.

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1.	Isolation of full-length human *HER2/neu*.

DNA oligonucleotide primers were designed that correspond to the DNA sequence of the wild-type form of *HER2/neu*. Two sets of overlapping primers were designed in order to amplify the large cDNA sequence in two manageable fragments. Sequences corresponding to the *HER2/neu* intracellular/transmembrane and extracellular/transmembrane regions were amplified from total RNA prepared from SKBR3 cells (a human mammary epithelial cell line obtained from ATCC that over-expresses *HER2/neu*). Amplified products (ECD=2082 bp and ICD=1724 bp, respectively) were isolated by gel electrophoresis and subcloned into plasmid PCR-Script SK+ (Stratagene). Resulting plasmid clones were analyzed by restriction digestions and sequenced by dideoxyflourescent-labeled sequencing methods to confirm fidelity of the clones. In order to join the ECD and ICD fragments into the full-length version of human *HER2/neu* for the eventual construction of the bicistronic retroviral vector (see below), a mutation was introduced into the ICD fragment plasmid to generate a unique restriction site (BspEI). This was done by the QuikChange mutagenesis kit (Stratagene) and appropriate primers. Since BspEI is sensitive to *dam* methylation both the mutated ICD plasmid and the existing ECD plasmid were sub-passaged through a *dam- e.coli* strain (SCS 110 from Stratagene). The ICD fragment was then ligated into the ECD plasmid. Diagnostic restriction enzyme digests and DNA sequencing revealed that the correct full-length human *HER2/neu* sequence was present and true in resulting clones. The resulting plasmid was called pCR-Script-*HER2/neu* FL.

2. Construction of a bicistronic recombinant retroviral vector for transfer of the *HER2/neu* and enYFP genes.

Next, the full-length *HER2/neu* sequence was subcloned into a shuttle vector (pSV-enYFP; Medin unpublished) containing the internal ribosome entry site (IRES) from the encephalomyocarditis virus along with the sequence for the enhanced yellow fluorescence protein (enYFP; Stratagene). Retroviral gene transfer vector pUMFG/*HER2/neu*/IRES (10.6 kb) was then constructed by transferring a NcoI/ClaI fragment containing the full length human *HER2/neu* cDNA from the pCR-Script-*HER2/neu* plasmid to the pSV/IRES/enYFP shuttle vector. A NcoI/NotI fragment containing the sequence *HER2/neu*/IRES/enYFP was then transferred into the retroviral vector pUMFG to give pUMFG/*HER2/neu*/IRES/enYFP. *HER2/neu* was again sequenced to fully re-confirm its fidelity.

3.	Transfection of retroviral producer cells to produce stable producer clones.

Amphotropic AM12 onco-retroviral packaging cells (Takenaka et al. 1999) were grown to 90% confluency in appropriate medium and transfected using a calcium phosphate method (Takenaka et al. 1999) with 0.7 ug of plasmid pGTN28, which confers resistance to neomycin, and 7 ug of plasmid pUMFG/*HER2/neu*/IRES/enYFP. Cells were incubated for 10 hrs at 37°C (5% CO_2) and then placed under

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G418 selection for two weeks. Cells were then flow sorted on a FACs Scan (BD) using appropriate conditions and controls (see below) to obtain producer cells highly expressing (top 6%) both *HER2/neu* and enYFP. These pooled positive producer cells were then expanded and stored frozen or used in infection experiments.

4. Flow cytometry analyses for *HER2/neu* and enYFP expression.

Transfected AM12 producer cells expressing *HER2/neu* and enYFP (AM12/*HER2/neu*/enYFP) were trypsinized, washed in DPBS containing 0.1% (w/v) D-glucose and 0.02% (v/v) EDTA (final pH adjusted to 7.2), and stained with 20ml of IgG1 isotype conjugated to PE or mouse α-hu*HER2/neu* directly conjugated to PE (Becton Dickinson) according to the manufacturer's specifications. 10,000 cell events were acquired on a FACScan flow cytometer and analyzed using CellQuest software (Becton Dickinson).

5. Productive infection of test cells and titer determination.

Naïve, non-transfected AM12, the pool AM12/*HER2/neu*/enYFP, and PG13/enYFP (Medin unpublished) producer cell lines were grown in 10 cm tissue culture plates to 90% confluency in 7 ml of complete medium. Test recipient cell lines, NIH3T3 and HeLa, were grown to 20% confluency in 10 cm tissue culture plates and counted on a hemocytometer using Trypan Blue dye exclusion (Bio-Whittaker) prior to infection. Producer cell line supernatants were prepared by aliquoting 4 ml of filtered supernatant and 6 ml of medium in the presence of protamine sulfate (final concentration of 8 µg/ml). This medium was subsequently transferred to recipient cells and incubated overnight (37°C, 5% CO_2). Recipient cells were gently washed twice and incubated for 48 hrs in 10 ml of complete medium, before being stained and analyzed by flow cytometry for *HER2/neu* and enYFP expression as described above. Virus packaged by the AM12/*HER2/neu*/enYFP producer pool was shown to have a titer of ~10^5 productive infectious units/ml on NIH3T3 and HeLa cells, respectively.

6. Tumor cell lines. SKBR3 and MCF-7 cells were maintained in our lab.

MCF7 P+3 cells were passaged <u>in vivo</u> and provided by Dr. Steven Swanson. MDA-MB-435 and MDA-MB-231 cells were obtained from Dr. Janet Price at the University of Texas M.D. Anderson Cancer Center. The MDA-MB-435 and MDA-MB-231 cell lines were isolated from pleural effusions of two patients with breast cancer. All three cell lines are estrogen-independent and express *HER2/neu* poorly relative to SKBR3. MCF-7 primary tumors are unable to yield metastases. In contrast, primary tumors of MDA-MB-435 cells can form spontaneous lymph node and lung metastases in 80-100% of nude mice injected with 10^5 to 10^6 cells into the mammary fat pad (mfp) at 12 weeks post-inoculation. Subcutaneous injection into the lateral flank, however, decreases the frequency of mice with metastases to 20-40%. Tail vein injection of 10^6 cells yields experimental lung metastases in ICR-SCID mice at a rate of 4/9 using non-transfected cells or 7/9 using *HER2/neu*-transfected cells. MDA-MB-231 cells can form primary tumors in nude mice injected with 10^5 to

10^6 cells into the mfp, but the frequency of mice developing macroscopic lung metastases 20 weeks post-injection is only 1/7. MDA-MB-231 cells have also been reported to generate lung metastases in nude mice following tail vein injections. MCF7 cells were maintained in MEM with Eagle's salts (Gibco) supplemented with 10% FCS (Gibco), 1% Penicillin/Streptomycin, 1.5 g/L of sodium bicarbonate, 0.1 mM non-essential amino acids for MEM, 1 mM sodium pyruvate (Bio-Whittaker), and 0.01mg/ml Bovine Insulin (Gibco). MDA-MB-435 and MDA-MB-231 cells were maintained in MEM supplemented with 5% FCS, 1% L-Glutamine, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids and vitamins for MEM (Sigma). SKBR3 cells were grown in DMEM complete: DMEM (Bio-Whittaker) complete with 10% FCS (Gibco), 1% penicillin/streptomycin (Gico), and 1% L-glutamine (Gibco).

7.   Transductions of tumor cell lines.

Producer cells AM12/*HER2/neu*/enYFP, AM12 non-transfected, and PG13/enYFP cells were grown to 90% confluency in 10-cm tissue culture plates in 10 ml of recipient cell medium. Recipient tumor cells were grown to 10 to 20% confluency in 10 ml of its respective medium as described above. Media on recipient cells were aspirated and replaced with 10 ml of 0.45 µm-filtered supernatant from plates of AM12/*HER2/neu*/enYFP, AM12 NT, or PG13/enYFP cells. 20 µl of 4µg/ml protamine sulfate were added to each recipient plate for a final concentration of 8 µg/ml. Infections of the same recipient cells were repeated daily until the recipient cells reached about 90% confluency, at which point the cells were split and incubated overnight before continuing the daily transductions. A total of 12 transductions were performed on all four target cell lines cells using supernatants taken from AM12/*HER2/neu*/enYFP and AM12 NT cells. A total of 5 transductions were performed for all four target cell lines using supernatant taken from PG13/enYFP cells. *HER2/neu*-transduced cells were stained with anti-hu*HER2/neu*-PE (BD Biosciences) and all cells were flow sorted on *HER2/neu* and enYFP to collect the top 3-20% *HER2/neu*- and/or enYFP- expressing cells. Flow cytometry analyses are shown below and indicate high levels of expression of both target antigens in all cell types.
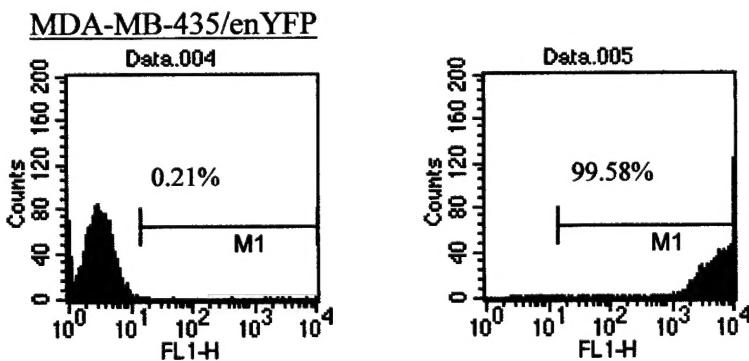
MDA-MB-435/enYFP

enYFP

MDA-MB-435/*HER2/neu*/enYFP

Data.020

0.04 | 0.17

90.28%

FL2-H (y-axis), FL1-H (x-axis)

Isotype

Data.021

6.89 | 92.88%

0.03

FL2-H (y-axis), FL1-H (x-axis)

HER2/neu

enYFP

MDA-MB-231/enYFP

Data.001

0.30%

M1

Counts (y-axis), FL1-H (x-axis)

Data.002

MFI=4341.37

90.25%

M1

Counts (y-axis), FL1-H (x-axis)

enYFP

MDA-MB-231/*HER2/neu*/enYFP

Data.016

0.58% | 1.95

87.09%

FL2-H (y-axis)

enYFP

FL1-H (x-axis)

Isotype

Data.017

3.28% | 91.02%

0.11%

FL2-H (y-axis), FL1-H (x-axis)

HER2/neu

MCF7/enYFP (unsorted)

Data.007

1.11% | 0.75%

8.13%

FL2-H (y-axis), FL1-H (x-axis)

MCF7P+3/enYFP (unsorted)

Data.014

2.75 | 1.82

11.02%

FL2-H (y-axis), FL1-H (x-axis)

enYFP

8

MCF7/*HER2/neu*/enYFP

Data.005

0.05%   0.32%

59.10'

FL2-H (y-axis: 10^0 10^1 10^2 10^3 10^4)
FL1-H (x-axis: 10^0 10^1 10^2 10^3 10^4)

Isotype →

Data.006

6.89

92.88%

0.03

FL2-H / FL1-H
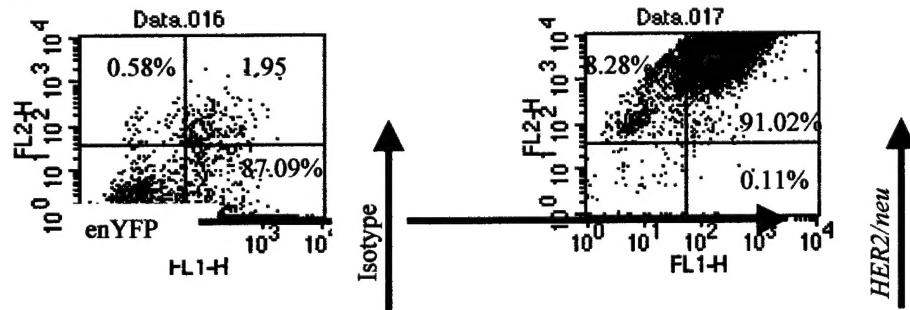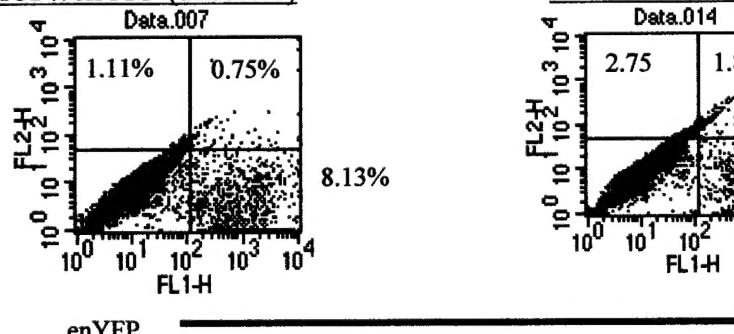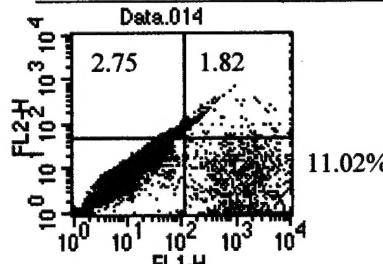
HER2/neu →

enYFP →

MCF7P+3/*HER2/neu*/enYFP

Data.012

0.08%   1.17%

68.85'

FL2-H / FL1-H

Isotype →

Data.013

40.30

36.22%

0.11

FL2-H / FL1-H
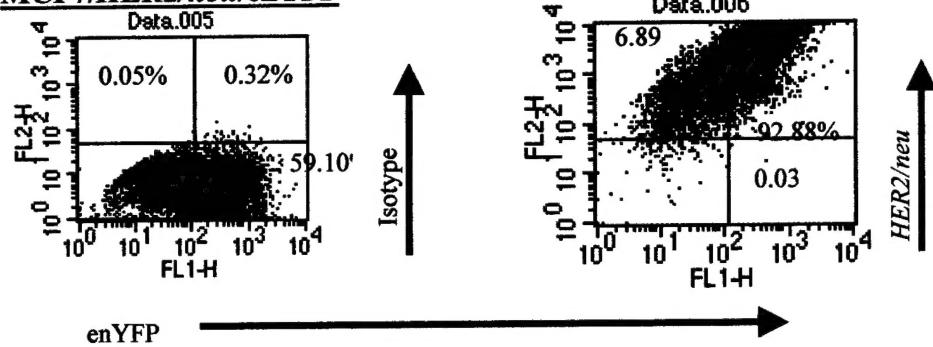
HER2/neu →

enYFP →
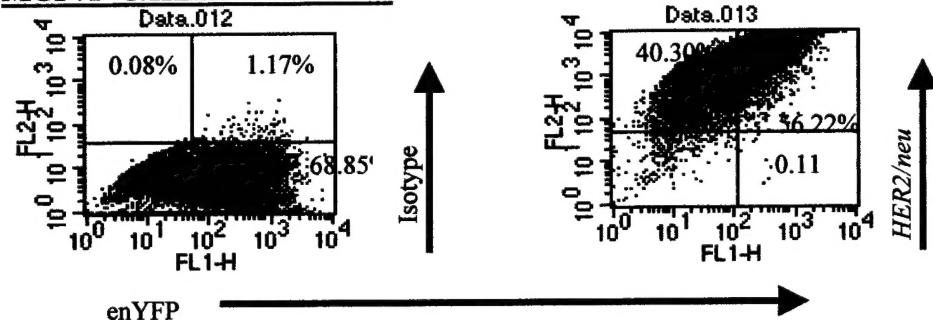
SKBR3 (reference cell line)

Data.029

0.93

M1

Counts (y-axis: 0 – 200) / FL1-H (10^0 10^1 10^2 10^3 10^4)

Data.030

89.82%

M1

Counts / FL1-H

→

****
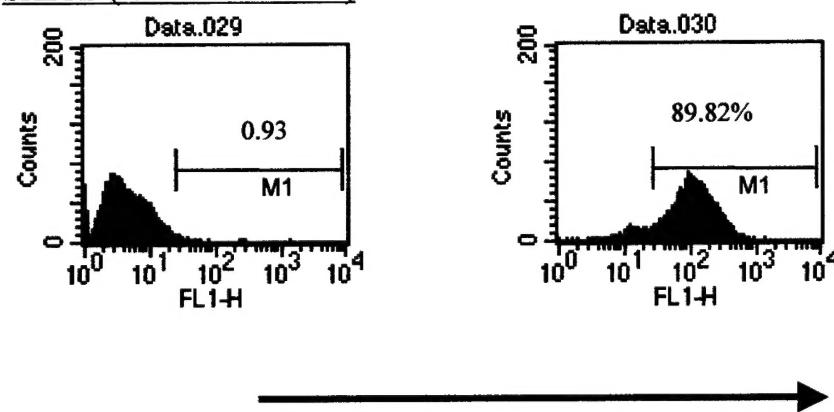
OBJECTIVE 3: Measure the biodistribution of labeled Herceptin in mice bearing human breast cancers.

****

Based on the observed in vitro binding data of RICs to normal expressing and over-expressing *HER2/neu* tumor cells (Objective 1), it was hypothesized that RICs will have similar in vivo binding patterns. There should be significant RIC accumulation in *HER2/neu* over-expressing tumor cells (BT-474) and not as much in normally expressing *HER2/neu* tumor cells (MCF-7) or in normal mouse cells (non-tumor tissues).

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Female nude mice weighing about 20 grams each, were used in these studies. Mice were inoculated with either MCF-7 tumor cells, which express normal amounts of *HER2/neu* membrane receptor protein or with BT-474 cells that over-express the receptor protein. Details concerning the production of xenografts can be found under Objective 3 in Exhibit B. When the tumors became palpable (about 0.5 cm or larger) the mice were injected intra-peritoneally (i.p.) with about 20 µl of [111]-In bound to either Herceptin® or to nonspecific mouse IgG immunoconjugate. The dosages ranged from about 3 to 30 µCi of ^{111}In-RIC containing 1 to 10 µg IC protein. Six mice were used for each determination. The injected mice were provided food and water <u>ad lib</u>, and housed in a special sterile environment for immunocompromised animals.

In the first set of biodistribution experiments, 6 nude mice bearing MCF-7 xenografts and 6 nude mice bearing BT-474 xenografts were injected with 2 to 4 µCi of [111]-In-Herceptin and sacrificed at 96 hours post i.p. injection. In addition, 6 nude mice bearing BT-474 xenografts were injected with 4.1 µCi of ^{111}In-IgG protein and sacrificed at 96 hours. The animals were euthanized with carbon dioxide. The blood was collected by heart puncture using heparinized 1-ml syringe. Three samples of blood and other tissues (tumor, liver, lung, heart, spleen, stomach, and kidney), each weighing about 50 mg (wet weight), were collected. Tissue samples were counted in glass gamma-counting tubes using a well gamma-counter (Ludlum Model 203 Shielded Well Scintillator). Each sample was counted for 10-20 seconds and values expressed, after back ground radioactivity correction, as CPM/mg tissue. Each experiment was repeated at least once. Biodistribution data are expressed as % of the injected dose per gram of tissue. The biodistribution data are presented in Figure 1 (A, B, and C).

Figure 1 (A)
Biodistribution of ^{111}In-Herceptin
(2.86 µCi/mouse, 96 hours)
## *6 nude* mice bearing MCF-7 xenografts
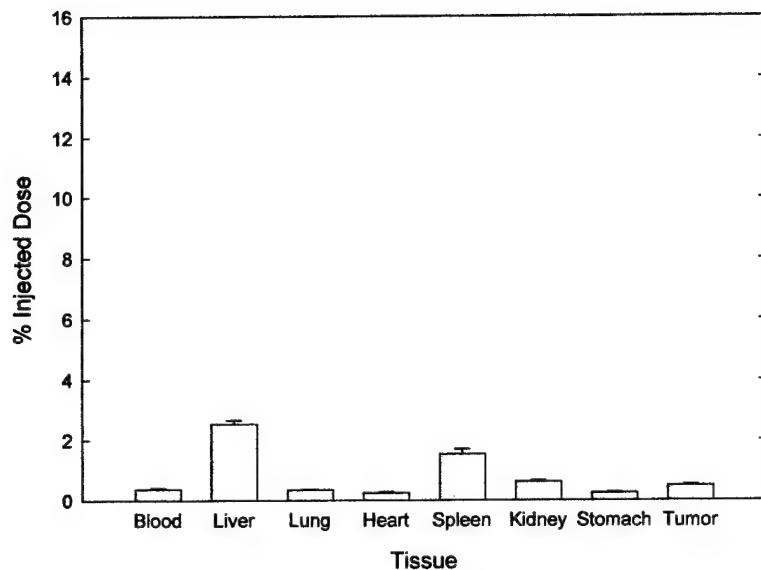
Figure 1 (B)
Biodistribution of ^{111}In-Herceptin
(2.51 µCi/mouse, 96 hours)
6 nude mice bearing BT-474 xenografts
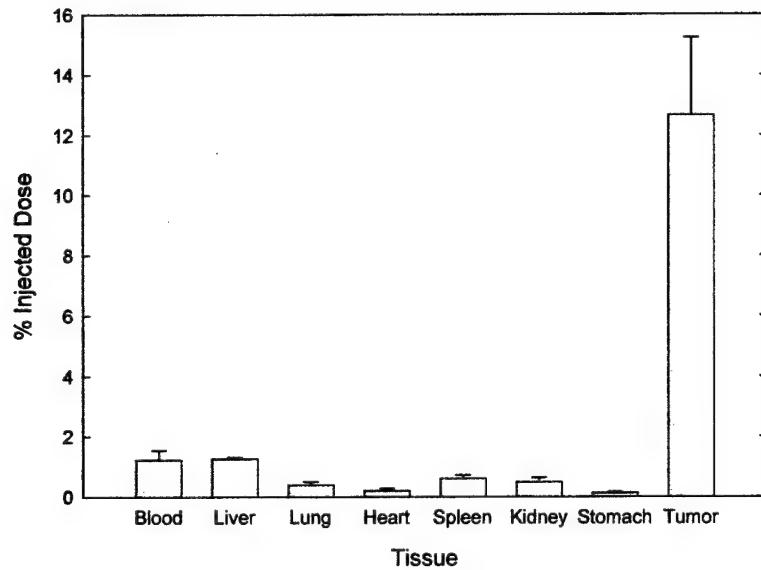
Figure 1 (C)
**Biodistribution of ^{111}In-IgG**
(3.82 µCi/mouse, 96 hours)
***6 nude* mice bearing BT-474 xenografts**
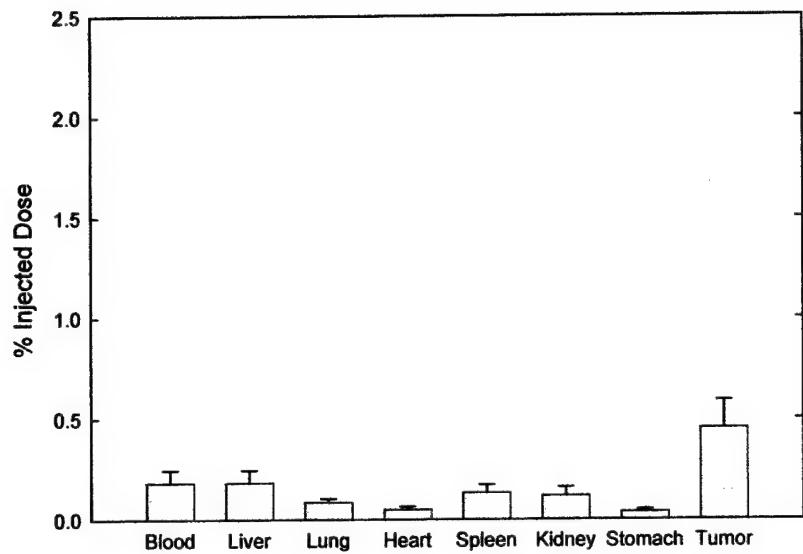
The pattern of tissue distribution of [111]-In-Herceptin in the three groups of mice is presented in Fig 1 (A, B and C). There is little uptake of labeled Herceptin® in MCF-7 tumor cells compared to BT-474 tumor cells (Fig 1 A and B). There is a clear difference in the distribution of labeled Herceptin® and IgG protein in tumors of BT-474 tumor-bearing mice and MCF-7 tumor-bearing mice. Nonspecific mouse ^{111}In-IgG is slightly more concentrated in the BT-474 tumor cells than in other tissues (Fig 1-C). However, labeled Herceptin® is concentrated more than 6-times in BT-474 tumors than in other organs of these mice (Fig 1B).

In the second series of biodistribution experiments, nude mice bearing MCF-7 and BT-474 tumor cells were sacrificed at 48, 72, and 96 hours post-injection. Six animals were sacrificed in each group at each time period for a total of 36 animals. The data from these studies are presented in Figure 2 (A and B).

FIGURE 2 (A)
Biodistribution of [111]-In Herceptin in
6 nude mice bearing MCF-7 Xenografts
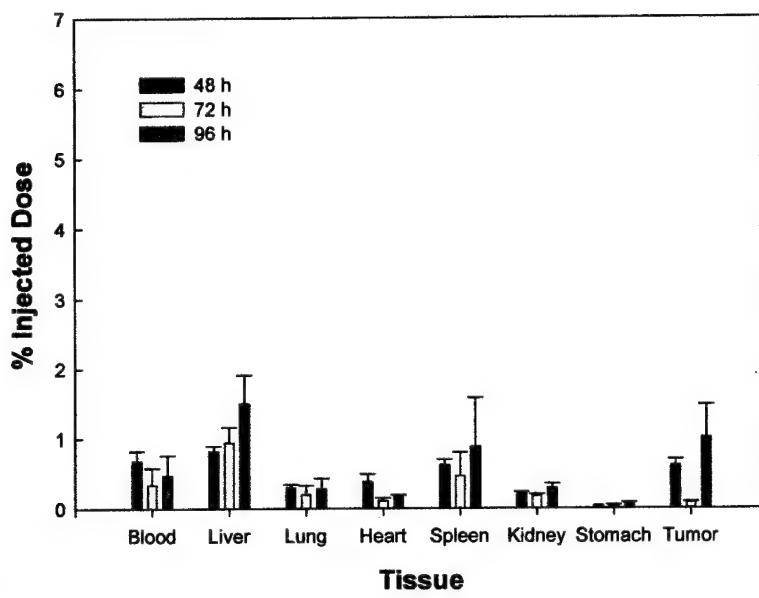
at 48, 72, and 96 hr post injection

FIGURE 2 (B)
Biodistribution of [111]-In Herceptin in
6 nude mice bearing BT-474 Xenografts
at 48, 72, and 96 hr post injection
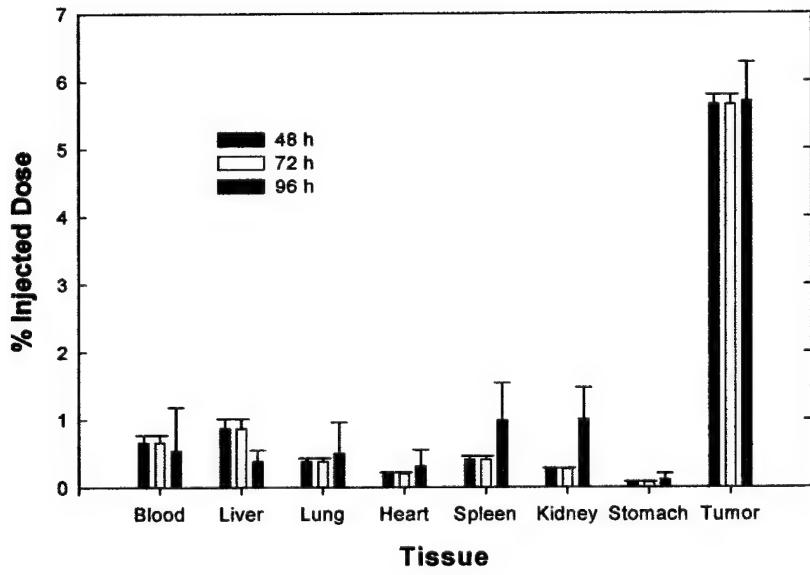
In mice bearing MCF-7 xenografts the [111]-In-Herceptin is retained in blood, liver, spleen, and tumor (Fig 2 A) and the concentration in these tissues increases with time. However, the increase in tumor cells was not as

consistent as in other tissues. In the case of BT-474 xenografted mice the highest level of radioactivity (~6-times) was seen in tumor cells within 24 hr after injection and remained high until the termination of the experiment (96 hr) (Fig 2 B).

****

OBJECTIVE 4: Evaluate [111]-In-Herceptin® as a tumor-imaging agent using nude mice xenografted with human cancer cells that over-express *HER2/neu*.

Sensitive imaging agent for advanced breast cancer patients with tumors that over-express *HER2/neu* may be useful for staging and assessing therapy. At 24, 48, 72, and 96 hours post i.p. injection, mice were anesthetized and imaged for 10 minutes with a Picker Prism 2000 gamma camera equipped with a dedicated Odyssey FX Computer System and medium-energy collimators. The camera was peaked for the two [111]-In gamma photons using a 10% window around each peak. The activity within each mouse was determined using the manufacturer's region of interest (ROI) program. Activity within the tumor tissue was determined with the same ROI program. Mean values from the ROI analysis for whole body and tumor tissues were used to construct whole body and tumor time-activity curves. Data obtained from each imaging session are presented as follows:

<div align="center">

TUMOR UPTAKE AS A %

OF TOTAL BODY COUNTS

</div>

Tumor	Time (hours)			
	24(n = 2)	48(n=2)	72(n=3)	96 (n=3)
MCF-7	9.1*	8.5	8.7	8.0
BT-474	----	28.1	26.1	12.5

* % mean counts in the tumor ROI divided by mean total counts in the whole body ROI.

IMAGE 1:

72 hour posterior image of 3 nude mice
with BT-474 xenografts on their backs.
Mice were injected with 5 µCi ^{111}In-Herceptin.
Note clear visualization of the tumors.
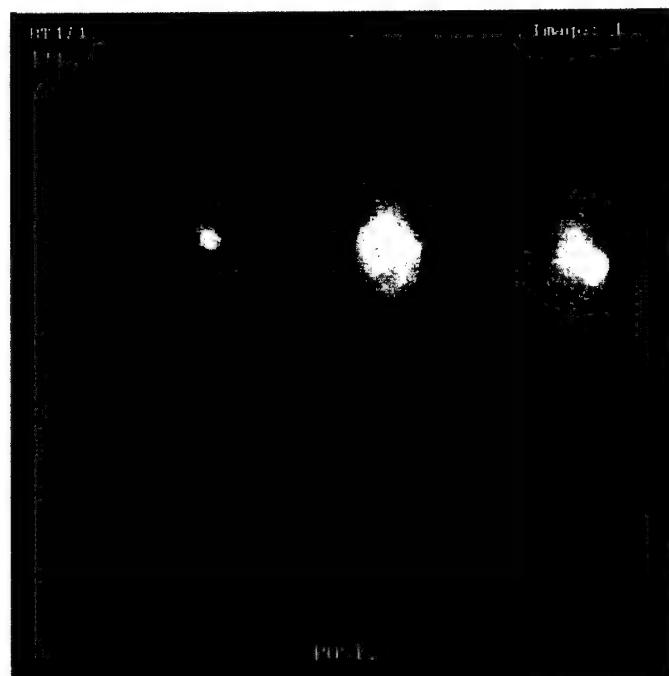
IMAGE 2:

72 hour posterior image of 2 nude mice
with MCF-7 xenografts on their backs.
Mice were injected with 5 µCi ^{111}In-Herceptin.
Note the tumors are not visualized.
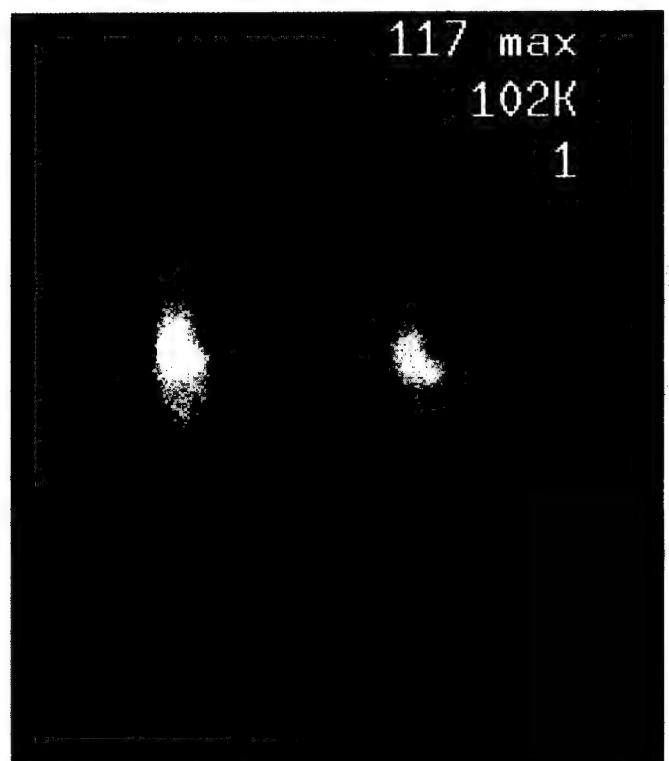
Region of Interest Images

BT-747   MCF-7
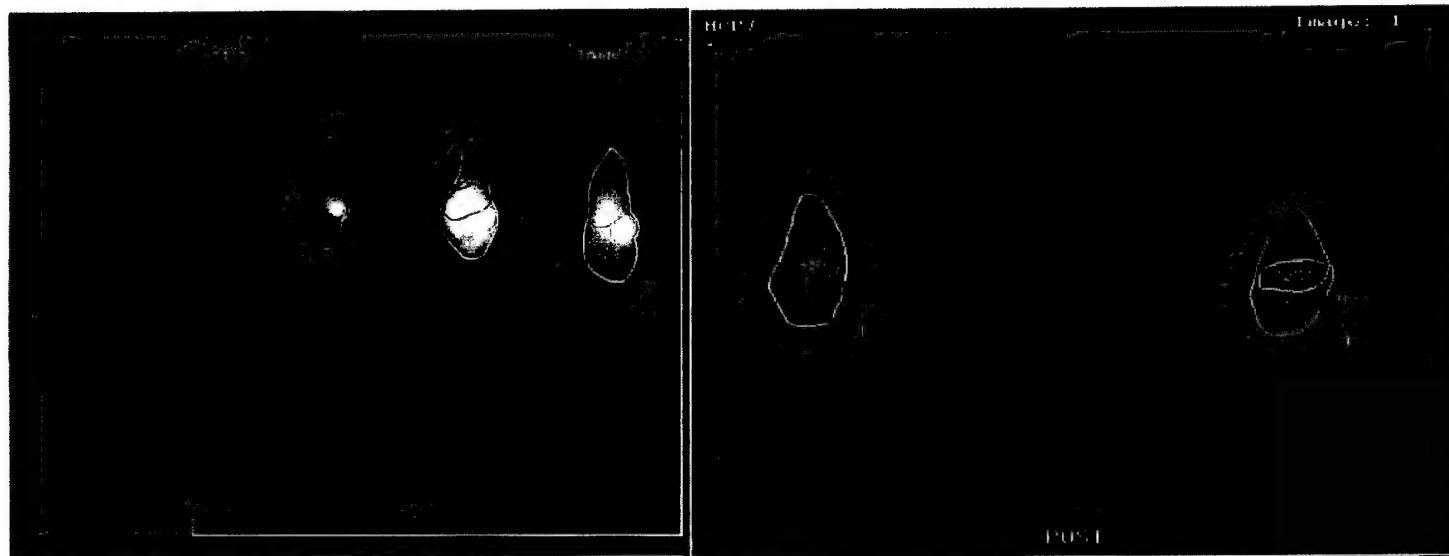
The data and images presented in this section clearly indicate that ^{111}In-Herceptin® accumulates in BT-474 tumor cells and not as much in MCF-7 tumors cells in vivo. The ratio of tumor to background uptake is > 6:1 which is adequate for most gamma camera system to detect tumor sites in the human body. This would suggest that ^{111}In-Herceptin can perform as an effective physiological imaging agent in women with *HER2/neu* over-expressing tumors.

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OBJECTIVE 5: Compare the anticancer efficacy of [90]Yttrium conjugated Herceptin to unlabeled Herceptin in nude mice bearing tumors of human breast cancer cells that over-express HER2.
****

The proposed studies in Objective 5 are presently underway and a "no-cost" extension will be requested to complete this portion of the study. Nude mice are being inoculated subcutaneously with MCF7 tumor cells or BT-474 cells. Once the tumors reach a volume of approximately 100 mm^3, the mice are randomized by weight and tumor size to various treatment groups. Nude mice are injected with ^{90}Y-Herceptin® ip once a week for 3 weeks. Treatment doses are 0.1, 1.0 and 10.0 mg/kg assuming 2.5 mCi/mg Herceptin®. Unlabeled Herceptin® is being injected into another set of mice with BT-474 xenografts at concentrations of 0, 1.0, 15, and 30 mg/kg. The effect of Herceptin® on the growth of BT-474 tumors will serve as a baseline control for the ^{90}Y-RIC studies. All mice are sacrificed 21 days after the first RIC injection. Final tumor measurements and necropsy

occurs on day 21. Tumors and organs are harvested and a portion of each tissue analyzed for radioactivity. A separate portion of each tissue is processed for histologic assessment of radiation necrosis. Differences in final tumor volumes between groups will be evaluated by single-factor ANOVA of the log-transformed data.

ACCOMPLISHMENT & REPORTABLE OUTCOMES:

During the first two years of our investigation we have accomplished the following:

1. Successful labeling of pharmacy-grade Herceptin® with ^{111}In using a new DTPA chelation method. A manuscript describing results has been accepted for publication.

2. Successful labeling of a purified monoclonal antibody directed against the *HER2/neu* oncoprotein with ^{111}In using a DOTA reagent.

3. Successful and significant <u>in vitro</u> binding of ^{111}In-Herceptin® to breast cancer cells that over-express the HER2/neu oncoprotein (receptor).

4. <u>In vitro</u> and <u>in vivo</u> stability of the new RIC (Herceptin-CHX-A'-DTPA-In[111]) has been demonstrated.

5. Development of a new breast tumor cell line which over-expresses *HER2/neu* receptor protein and contains a genetic marker so that metastatic lesions can be followed.

6. Successful biodistribution and imaging studies of tumor bearing mice with MCF-7 and BT-474 tumors.

CONCLUSIONS:

We conclude that our work so far demonstrates proof-of-hypothesis for Objectives 1, 2, 3, and 4. Pharmacy grade Herceptin® can be labeled with ^{111}In using a DTPA chelation procedure (to our knowledge this observation has not been published before) and that it binds to tumors cells <u>in vitro</u> and <u>in vivo</u>. There is significantly more binding to tumors that over-express HER2/neu than to those tumors that express normal concentrations of HER2/neu receptor.

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APPENDICES

Following are copies of published abstracts reporting the results of this investigation. Also attached is a copy of the editor's proof of a full paper about to be published.

Abstract presented at the 2002 Southern Ontario Gene Therapy meeting at Langdon Hall in Cambridge, Ontario, on April 21&22, 2002.

Immuno-gene therapy against prostate cancer using Her2/neu as a model antigen. Miriam Mossoba, Christopher Siatskas and Jeffrey A. Medin. Department of Medical Biophysics. University of Toronto.

Abstract

Prostate cancer is the second leading cause of cancer-related death in men in North America. Standard treatments for prostate cancer include chemotherapy, radiotherapy, androgen ablation, and prostatectomy. Better treatment modalities are needed to effectively eradicate/treat/contend with metastases. Efforts to harness the power of the immune system in recognizing and eliminating prostate tumors using antigen loading of dendritic cells have been met with limited success. Transferring tumor associated antigens genes directly into dendritic cells (DCs) overcomes some difficulties involved in antigen loading. In the present study, a prostate tumor associated antigen gene, *Her2/neu*, has been sub-cloned and incorporated into an MFG-based oncoretroviral vector. Her2/neu antigen is an EGF-R-related transmembrane tyrosine kinase that is an attractive target for immuno-gene therapy becuase it is selectively overexpressed in 20% of prostate tumors and 80% of its metastases in men. Vectors have been constructed that engineere expression of *Her2/neu* alone. Eventually, a cytokine, a co-stimulatory factor, or a fluorescent protein coding sequence will be added downstream of *Her2/neu* in a series of bicistronic vectors that may further potentiate effects or facilitate marking/detection. *Ex vivo* transduction of C57BL/6 mouse DCs will be performed. Transduced DCs will be injected into mice harboring Her2/neu-overexpressing prostate tumors. Tumor sizes as well as CTL and antibody responses against Her2/neu will be measured to determine the efficacy of this approach. Preliminary results show that the completed vector construct carrying the full length *Her2/neu* sequence alone yields cell surface expression in a producer cell line.

LABELING HERCEPTIN® WITH ^{111}In and ^{90}Y AND UPTAKE STUDIES IN MCF-7 AND SKBR-3 CELL LINES.

M. J. Blend, J. J. Stastny, S. M. Swanson, University of Illinois, Chicago, IL 60612 and **M. W. Brechbiel**, ROB, NCI, NIH, Bethesda, MD 20892.

Objectives: The goal of this research was to develop stable radioimmunoconjugates (RICs) of Herceptin® (H) for radioimmunoscintigraphy and radioimmunotherapy in an animal model bearing human breast tumors. H is used clinically for the treatment of breast cancer patients whose tumors over-express HER2/neu receptor. **Methods**: Pharmacy-grade H was conjugated with a DTPA linker in preparation for labeling. ^{111}In-Cl and ^{90}Y-Cl were labeled to H-CHX-A''–DTPA according to the procedure of Clarke *et al.* (*Cancer Res* 60: 4804, 2000). The binding capacity of ^{111}In-RIC was tested with human cancer cell line MCF-7 which expresses normal amounts of HER2/neu receptor and SKBR-3 cells which over-express HER2/neu receptors. **Results**: The molar ratio of DTPA-CHX-A'' to H in the tested immunoconjugate was 1.4:1. Using a molar excess of 10:1 CHX-A''-DTPA to H, a specific activity of 1.87 μCi ^{111}In/μg H-RIC and 2.71 μCi ^{90}Y/μg H-RIC was obtained. The purity of RIC was 96%+ for ^{111}In and 99%+ for [90]Y. Stability at 37° C for both RICs ranged from 98% at 24 hours in human plasma to 85% at 96 hours. [111]In- RIC bound to MCF-7 cells with a binding radio of 2.5:1 using a nonspecific IgG RIC as a control. A 98.3:1 binding ratio was observed when SKBR-3 cells were exposed. **Conclusion**: H can be labeled with ^{111}In and ^{90}Y using a CHX-A''-DTPA linker. Stable RICs are formed. To our knowledge, this is the first time that H has been labeled with ^{111}In or ^{90}Y using this DTPA conjugate. There is significant cell binding of [111]-In-RIC when HER2/neu over-expressing cells are used in culture. Animal *in vivo* and *in vitro* studies using ^{111}In and ^{90}Y-CHX-A''-DTPA-H are underway.

. Following 3 abstracts were presented at the Central Chapter Meetings of the Society of Nuclear Medicine in March 2002 and will be published ·in Clinical Nuclear Medicine in 2002.

LABELING HERCEPTIN® AND anti-HER2/neu ANTIBODY WITH IN-111 USING DTPA AND DOTA CONJUGATES. <u>M. J. Blend</u>, J. J. Stastny, S. M. Swanson, University of Illinois, Chicago, IL.

Objectives: The goal of this research was to develop stable radioimmunoconjugates (RICs) of Herceptin® (H) and an anti-HER2/neu antibody (anti-HER MoAb) for tumor localization in an animal model bearing human breast tumors. Methods: Pharmacy-grade H and pure anti-HER2/neu MoAb were conjugated with a DTPA and DOTA linker respectively. In-111-chloride was labeled to H-CHX-A''–DTPA (linker supplied by Dr. M. W. Brechbiel, NIH/NCI) according to the procedure of Clarke et al. (*Cancer Res* 60: 4804, 2000). In-111-chloride was labeled to *anti*-HER2/neu MoAb DOTA complex (Li *et al., Bioconjug Chem.* 4: 275, 1993). Results: The molar ratio of DTPA-CHX-A" to H in the tested immunoconjugate was 1.4:1. Using a molar excess of 10:1 CHX-A''-DTPA to H, a specific activity of 1.87 µCi In-111/µg H (RIC) was obtained. RIC purity was 96.8%. H-RIC in sodium acetate solution (SAS) and human plasma (HP) at room temperature (RT) and 37° C (body temperature) was quite stable. At 24 hr. the stability of H-RIC was 97.9% at RT and 98.1% at 37° C in SAS. The stability of H-RIC was 95.9% at RT and 95.5% at 37° C in HP. At 96 hr. the stability of H-RIC was 95.7% at RT and 94.8% at 37° C in SAS and in HP, 90% at RT and 85% at 37° C. HER2/neu MoAb DOTA complex was successfully labeled with a purity of 95.5% and a specific activity of 1.45 µCi In-111/µg RIC. Conclusions: H can be labeled with In-111 using a CHX-A''-DTPA linker to provide a stable RIC. To our knowledge, this is the first time that H has been labeled with In-111 using this DTPA conjugate. Animal <u>in vivo</u> and <u>in vitro</u> studies using In-111-CHX-A"DTPA-H are underway.

IN VITRO CELL BINDING AND KILLING OF HUMAN BREAST CANCER CELLS WITH IN-111/Y-90 LABELED HERCEPTIN®.

M. J. Blend, J. J. Stastny, S. M. Swanson, University of Illinois, Chicago, IL

Objectives: The goal of this research was to develop stable radioimmunoconjugates (RICs) of Herceptin® (H) for tumor imaging therapy in an animal model bearing human breast tumors. H is used clinically for the treatment of patients whose breast tumors over-express HER2/neu receptor. **Methods**: Pharmacy-grade H was conjugated with a DTPA linker (supplied by Brechbiel, NIH/NCI) in preparation for labeling. In-111 and Y-90 chloride were labeled to H-CHX-A''–DTPA according to the procedure of Clarke et al. (*Cancer Res* 60: 4804-4811, 2000). The binding capacity of In-111-RIC was tested with human cancer cell line MCF-7 which expresses normal amounts of HER2/neu receptor and SKBR-3 cells which over-express HER2/neu receptors. The killing capacity of Y-90-RIC was tested in MCF-7 and BT-474 cells (HER2/neu over-expressers). **Results**: The molar ratio of DTPA-CHX-A'' to H in the tested immunoconjugate was 1.4:1. Using a molar excess of 10:1 CHX-A''-DTPA to H, a specific activity of 1.87 μCi In-111/μg H and 2.71 μCi Y-90/μg RIC was obtained. In-111-RIC bound to MCF-7 cells with a ratio of 2.5:1. There was a 98.3:1 binding ratio with SKBR-3 cells. At a concentration of 1.2 μCi Y-90-RIC/ml, 6.1 % of the MCF-7 cells and 66.7% of BT-474 cells were killed after 3 hours of incubation. **Conclusion**: H can be labeled with In-111 and Y-90 using a CHX-A''-DTPA linker. There is significant cell binding of In-111-RIC when HER2/neu over-expressing cells are used in culture. There is significant cell killing of BT-474 cells by Y-90-RIC in culture. Animal *in vivo* and *in vitro* studies using In-111/Y-90-CHX-A''-DTPA-H are underway.

LABELING HERCEPTIN® WITH YTTRIUM (Y-90) USING A DTPA CONJUGATE. <u>M. J. Blend</u>, J. J. Stastny, S. M. Swanson, University of Illinois, Chicago, IL

Objectives:: The goal of this research was to develop a stable radioimmunoconjugate (RIC) of Herceptin® (H) for radioimmunotherapy in an animal model bearing human breast tumors. H is used clinically for the treatment of patients whose breast tumors over-express HER2/neu receptor. **Methods**: Pharmacy-grade H was conjugated with a DTPA linker (linker supplied by Dr. M. W. Brechbiel, NIH/NCI) in preparation for labeling. Y-90-chloride was labeled to H-CHX-A''–DTPA according to the procedure of Clarke et al. (*Cancer Res* 60: 4804, 2000). **Results**: The molar ratio of DTPA-CHX-A'' to H in the tested immunoconjugate was 1.4:1. Using a molar excess of 10:1 CHX-A''-DTPA to H, a specific activity of 2.71 µCi Y-90/µg H RIC was obtained. RIC purity was 99.3%. Y-90 labeling efficiency was 27.1%. Y-90-H-RIC in phosphate buffered saline (PBS) at pH 7.4 and in human plasma (HP) at room temperature (RT) and 37° C (body temperature) was quite stable. At 24 hr. the stability of Y-90-H-RIC was 97.7% at RT and 97.0% at 37° C in PBS. The stability of Y-90-H-RIC was 97.6% at RT and 95.4% at 37° C in HP. At 96 hr. the stability of Y-90-H-RIC was 95.6% at RT and 81.5% at 37° C in PBS and in HP, 86.3% at RT and 80.5% at 37° C. **Conclusion**: H can be efficiently labeled with Y-90 using a CHX-A''-DTPA linker to provide a stable RIC. To our knowledge, this is the first time that H has been labeled with Y-90 using this DTPA conjugate. Animal <u>in vivo</u> and <u>in vitro</u> studies using Y-90-CHX-A''-DTPA-H are underway.

Biodistribution and Imaging of ^{111}In-Herceptin® in Immunodeficient Mice Bearing Human Breast Tumor Xenografts that Express Either Normal or High Levels of *HER2/neu* Receptor. S. M. Swanson, G, Yang, M.A.Q. Khan and M. J. Blend, University of Illinois at Chicago, Chicago, Illinois 60612

The goal of this research was to develop stable radioimmunoconjugates (RICs) of *anti-HER-2/neu* monoclonal antibodies (MoAbs) for imaging in an animal model bearing human breast tumors. Pharmacy-grade Herceptin® was conjugated with a CHX-A" DTPA linker and labeled with [111]InCl$_3$ according to the procedure of Clarke et al. (*Cancer Res* 60: 4804, 2000). Female nude mice weighing about 20 g each were inoculated with either normal *HER2/neu* expressing tumor cells (MCF-7 cells) or *HER2/neu* over-expressing tumor cells (BT-474 cells). When tumors reached ~ 2 to 5 mm diameter, mice (N=6/group) were injected (i.p., 2 to 4 μCi) with [111]-In-Herceptin® (MCF-7 & BT-474 bearing mice) or [111]-In-non-specific-IgG protein (BT-474 bearing mice only). All mice were imaged/sacrificed at 96 hours post RIC injection. Three samples of blood, tumor, liver, lung, heart, spleen, stomach, and kidney, each weighing ~50 mg (wet weight), were collected. There was little uptake of labeled Herceptin® in MCF-7 tumor cells (1% injected dose) in contrast to BT-474 tumor cells (14% injected dose). There was a clear difference in the distribution of labeled Herceptin® and non-specific IgG protein in tumors of BT-474 and MCF-7 tumor-bearing mice. [111]-In-Herceptin® concentrated 6-times more in BT-474 tumors than in any other tissues while ^{111}In-non-specific IgG was not. Corresponding 96 hr whole body images of these mice confirm the biodistribution results. These data demonstrate that pharmacy-grade [111]-In-Herceptin® can be preferentially and specifically concentrated in *HER2/neu* over-expressing human breast tumor *in vivo*. These tumors can also be successfully imaged at 96 hours post injection.

Labeling *anti-HER2/neu* Monoclonal Antibodies with ^{111}In and ^{90}Y using a Bifunctional DTPA Chelating Agent

(AU2)→Michael J. Blend,[1] Jerry J. Stastny,[1] Steven M. Swanson,[2] and Martin W. Brechbiel[3]

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(AU3)→*The goal of this investigation was to develop stable radioimmunoconjugates (RICs) of anti-HER2/neu monoclonal antibodies (MoAbs) for imaging and therapy in an animal model bearing human breast tumor xenografts that express normal (MCF-7 cells) and increased amounts of HER2/neu receptors (HCC-1954, BT-474, SKBR-3 cells) and on their cell surface membranes. Pharmacy-grade Herceptin®, a murine* ← (QU1) *anti-HER2/neu monoclonal antibody, and nonspecific mouse IgG protein were conjugated with the recently developed DTPA linker known as CHX-A—DTPA. These immunoconjugates were labeled with $^{111}InCl_3$ and $^{90}YCl_3$ (Wu et al., 1997. Bioconj. Chem, 5:1925) using a modification of a method published previously (Nikula et al., 1995. Nucl. Med. Biol. 22:387). Using a molar excess of 10:1 CHX-A -DTPA to immunoglobulin, average specific activities of 1.87 µCi ^{111}In/µg RIC and 2.71 µCi ^{90}Y/µg RIC were obtained. The purity of RICs was 96%+ for ^{111}In and 99%+ for ^{90}Y. Stability in human plasma at 37 . . . C for both RICs ranged from 98% at 24 h to 85% at 96 h. Binding capacity of the RICs was tested with human cancer cell lines MCF-7, HCC-1954, BT-474, and SKBR-3. Using ^{111}In-labeled nonspecific IgG protein as a control, ^{111}In-Herceptin® RIC was found to bind to MCF-7 cells with a ratio of 2.5:1 and to SKBR-3 cells with a ratio of 85:1 after 3 h of incubation. ^{111}In-anti-HER2/neu RIC bound to MCF-7 cells with a ratio of 6:1 and to SKBR-3 cells with a ratio of 115:1 after 3 h of incubation. ^{90}Y-anti-HER2/neu RIC bound 10-times greater to BT-474 cells than to MCF-7 cells. Thus, these MoAbs can be labeled with ^{111}In and ^{90}Y using the CHX-A -DTPA linker. The resulting RICs (^{111}In- and ^{90}Y-anti HER2/neu antibodies) are stable and bind significantly to HER2 overexpressing tumor cell lines.*

(AU4)→

INTRODUCTION

Women often present to their physician with locally advanced or metastatic breast cancer. A portion of these patients may have a particularly aggressive form of this disease with little or no clinical signs. Biopsy evaluation, as well as the measurement of serum tumor markers, hormone receptor status, and certain epidermal growth receptor status on the surface of tumor cells, help to determine the aggressiveness of a patient's disease. Between 25 and 30% of all breast cancer patients have an aggressive form of breast cancer known as *HER2/neu* positive disease.[1] The presence of *HER2/neu* overexpression signifies a poor prognosis for these patients.[1–4]

Immunotherapy with intact monoclonal antibodies (MoAbs) has been shown to be an effective method for treating patients with advanced breast cancer and low-grade non-Hodgkin's lymphoma. Recently the FDA approved an injectable MoAb (Herceptin®) that can slow the progress of *HER2/neu* overexpressing breast cancers in patients. In clinical trials, the *anti-HER2/neu* MoAb Herceptin® (trastuzumab, Genentech) showed significant biostatic activity both as a single agent

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and in combination with traditional cytotoxic chemotherapy in the treatment of *HER2/neu* overexpressing metastatic breast cancer patients.[5] Herceptin® is a recombinant *anti-HER2/neu* MoAb that targets the extracellular domain of HER-2 growth factor receptor, inhibiting signal transduction and cell proliferation. It is also thought to enhance antibody-dependent, cell-mediated cytotoxicity.[6,7]

Radioimmunotherapy (RIT) has been shown to deliver effective systemic tumor-targeted radiation for hematologic malignancies, particularly in non-Hodgkin's lymphoma patients. Yttrium-90 is a useful radionuclide for RIT due to its energetic beta emissions, ready availability at moderate cost, and absence of gamma emissions.[8] Adding the diagnostic isotope Indium-111 (^{111}In) to *anti-HER2/neu* MoAbs may convert these antibodies into effective physiologic staging (imaging) tools. Adding the therapeutic isotope Yttrium-90 (^{90}Y) to *anti-HER2/neu* MoAbs may improve their cytotoxic efficacy without adding significant risk to the patient.

The purpose of this paper is to describe the results of labeling *anti-HER2/neu* [including Herceptin®] antibody conjugates with the linker CHX-A -DTPA in preparation for future animal and human studies.

MATERIALS AND METHODS

Immunoglobulins

Herceptin® is a recombinant DNA-derived humanized MoAb of the IgG_1 kappa class that contains human framework regions with the complementarity-determining regions of a murine antibody (4D5) that binds to HER2 receptors. Herceptin®, as a purified preparation, was not available to us. We were able to obtain pharmacy grade Herceptin® from the hospital pharmacy. A vial of pharmacy grade Herceptin® contains a sterile, white to pale yellow lyophilized powder containing 440 mg trastuzumab, 9.9 mg L-histidine HCl, 6.4 mg L-histidine, 400 mg *alpha, alpha*-trehalose dihydrate, and 1.8 mg polysorbate 20, UPS.[9]

Anti-HER2/neu (pure) is a monoclonal antibody composed of mouse IgG_1 heavy chains and kappa light chains and was obtained from Becton Dickinson of San Jose, CA (Cat. # 340553). The antibody is diluted in 10 mM phosphate buffered saline (PBS, 0.15 M NaCl, pH 7.4 containing 0.01% sodium azide). Nonspecific mouse

IgG protein was obtained from BIO RAD Corporation (Richmond, CA).

Chelators

The bifunctional chelating agent 1,4,7,10-tetraazacyclododecane-N, N', N'', N'''tetraacetic acid (p-SCN-Bz-DOTA) was obtained from MACROCYCLICS, Inc., Richardson, TX (stock # B205). A recently developed bifunctional metal ion chelating agent 2-(p-nitrobenzyl)-*trans*-CyDTPA (CHX-A -DTPA) was provided by Dr. Martin Brechbiel of the NCI/NIH.

Isotopes

High purity ^{111}Indium chloride [Indiclor®] was obtained from Medi-Physics, Inc., Amersham Healthcare (Arlington Heights, Il.). It is supplied as a sterile solution of $^{111}InCl_3$ in 0.04 N HCl (pH = 1.4) with a specific activity of > 400 mCi/μg (carrier free) and a radiochemical purity of > 95%. High purity Yttrium-90 chloride was obtained from Perkin Elmer Life Sciences (Boston, MA) as $^{90}YCl_3$ in ~0.05N HCl with a specific activity of ~500 mCi/μg (carrier free) and a radiochemical concentration of ~ 500 mCi/mL at the time of production.

Human Breast Cancer Cell Lines

Human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 human breast cancer cells express a normal amount of *HER2/neu* receptors whereas the SKBR-3 cells overexpress *HER2/neu* receptors. Additional cell lines used in this study that overexpress *HER2/neu* receptors were HCC-1954 and BT-474.

Conjugation of Immunoglobulins

One hundred to 200 μg of immunoglobulin (Herceptin®, *anti-HER2/neu* and nonspecific mouse IgG) at a concentration of about 100 μg/mL were dialyzed against 3 L of 50 mM sodium bicarbonate buffer in 0.15 M NaCl, pH 8.0 (Buffer A), in the dark at room temperature for 18 h. The dialyzed immunoglobulins were then mixed with one of the two chelating agents in a molar excess of 10:1 and incubated in the dark at room temperature for 18 h. Unreacted (free) chelator was removed by dialysis against 3 L of Buffer A.

Two separate preparations of Herceptin® immunoconjugate were made to determine the variation in specific activity and labeling efficiency.

Pharmacy grade Herceptin® preparation was transferred to a dialysis system with a molecular cutoff of 10,000 Daltons and dialyzed overnight at room temperature against 3 L of Buffer A. After dialysis, total protein was determined by the BIO-RAD Protein Assay Kit (Richmond, CA) using bovine IgG (2 mg/mL) as a standard.

CHX-A -DTPA was added to the dialyzed Herceptin® in molar excess of 10:1 (Preparation A) and 50:1 (Preparation B) and incubated overnight at room temperature in the dark. CHX-A -DTPA-Herceptin® conjugate was mixed with an equal volume of 0.5 M sodium acetate buffer, pH 5.5, and stored at 4°C until use. The amount /number of bifunctional DTPA-CHX-A" ligand molecules bound to the Herceptin® molecule was determined using the method of Pippin et al.[11] Briefly, the total amount of IgG protein in the final preparation was determined with the BIO-RAD Protein Assay Kit. Arsenazo III-Yttrium (ARS-Y) solution was prepared from 5 mM Arsenazo III (ICN Pharmaceuticals, Inc., Costa Mesta, CA) and 0.1 M yttrium chloride (Sigma-Aldrich Fine Chemicals, St. Louis, MO). A working solution containing 5 μM Arsenazo III and 1.6 μM yttrium chloride in 0.15 M sodium acetate buffer, pH = 4.0, was used. To determine the molar ratio of DTPA to Herceptin®, the absorbance of the ARS-Y solution was measured at 652 nm using a Beckman DU-7 UV Spectrophotometer.

Labeling Immunoglobulins with [111]In Using a DOTA Linker

The procedure described by Li et al.[10] was used to label DOTA-immunoconjugates. A mixture of 100 μL of 0.3 mM p-SCN-Bz-DOTA in 0.2 M tetramethylammonium acetate (pH 5.0) and [111]InCl$_3$ (~1 mCi) were incubated at 37°C for 30 min. Unreacted (free) [111]In was removed by the addition of 25 μL of 5 mM DTPA in 0.1 M tetramethylammonium acetate (pH 6.0), followed by DEAE ion-exchange chromatography. The purified p-SCN-Bz-DOTA-^{111}In solution was mixed with approximately 300 μg of Herceptin® in 0.1 M tetramethylammonium phosphate (pH 9.0) and incubated at 37°C for 60 min. Radioimmunoconjugate was then isolated using a PD-10 column.

Multiple experiments using immunoconjugates containing the bifunctional chelating agent 1,4,7,10-tetraazacyclododecane-N, N′, N, N tetraacetic acid (p-SCN-Bz-DOTA) and [111]InCl$_3$

were performed. The specific activity (Ci ^{111}In/g RIC), radiochemical purity (% [111]In in RIC), and efficiency of labeling (% [111]In bound to RIC) were measured. The same procedure was used to label nonspecific mouse IgG protein. The radiolabeled immunoconjugates were purified by PD-10 column chromatography [PD-10 Sephadex®, G-25M Column, 3.1 inch (Supelco; Sigma, St. Louis, MO, Lot # 289348)] using an eluant 20 mM sodium acetate buffer pH 6.3 in 0.15 M sodium chloride.

Labeling Immunoconjugates with [111]In Using a DTPA Linker

Immunoglobulin labeling with ^{111}In and ^{90}Y was achieved via a bifunctional metal ion chelation agent, CHX-A -DTPA[12] using a modification of a method published previously.[13] ^{111}In-chloride was added to immunoconjugate at a concentration of 1 mCi per 100 μg Herceptin® RIC. The mixture was incubated for 20 min at room temperature and then the pH was adjusted to 7 with 2.0 M sodium acetate (pH 9.5). Excess ligand was removed by dialysis against 0.15 M sodium acetate pH 6.3 in 0.15 M sodium chloride. The RICs were separated from CHX-A-DTPA-^{111}In and free [111]InCl$_3$ by PD-10 column (Supelco; Sigma, St. Louis, MO) as follows. The PD-10 column was first washed with 15.0 mL of 1% human serum albumin in 20 mM sodium acetate buffer (pH 6.3) in 0.15 M sodium chloride and then with 15.0 mL of the same buffer without human serum albumin. The radiolabeled mixture was then loaded on the PD-10 column and fractions of 0.7 mL each were eluted with 20 mM sodium acetate buffer, pH 6.3, in 0.15 M sodium chloride. The radioactivity of each fraction was measured in a gamma well counter. The radioimmunoconjugates were eluted first. Purity of RIC was determined by instant thin layer chromatography (ITLC).

ITLC of the purified RIC was performed using glass fiber strips impregnated with Silica Gel [ITLC® SG, Pall Corporation, Ann Arbor, MI]. Strips (0.5 × 2.5 cm) were developed in a solvent system containing 10 mM EDTA (pH 4.5) and 10 mM NaOH in 0.9% saline. The area containing the RIC was located by cutting the strips and counting the radioactivity in each piece in a well counter. RIC counts were expressed as percent of the total ITLC strip radioactivity. The yield, purity, and specific activity of the ^{111}In-labeled RICs were measured for each immunoconjugate labeled.

Table 1. Specific Activity, Purity, and Efficiency of [111]In Labeling of *Anti-HER2/neu* and IgG p-SCN-Bz-DOTA Conjugate

Protein	Specific activity $\mu Ci/\mu g$	Purity of RIC (%)	Efficiency of labeling (%)
Herceptin®-DOTA	2.64	89.6	37.3
Anti-HER2/neu-DOTA	1.55	97.7	14.3
IgG-DOTA	1.45	96.5	13.7

Labeling Immunoconjugates with [90]Y

The immunoconjugate (CHX-A —DTPA-antibody) was labeled with $^{90}YCl_3$ according to the procedure of Wu et al.[12] using a modification of a method published previously.[13] Five hundred Ci of $^{90}YCl_3$ was added to 50 g of immunoconjugate and incubated in a glass vial at room temperature for 20 min. The reaction was terminated by adding 40 μL of 2 M sodium acetate buffer to bring the pH to 7.0. The mixture was then transferred to a PD-10 column for separation. The column was first eluted with 2 mL of 20 mM sodium acetate buffer pH 6.3 in 0.15 M sodium chloride and then four fractions (0.7 mL each) of the eluant were collected. The first fraction to show a high level of radioactivity usually contained the radioimmunoconjugate. The specific activity (Ci ^{90}Y/ g RIC), radiochemical purity (% ^{90}Y in RIC), and efficiency of labeling (% ^{90}Y bound to RIC) were measured as described earlier. The same procedure was used to label and purify nonspecific mouse IgG protein.

Stability of RICs

In vitro stability of the ^{111}In-RIC and ^{90}Y-RIC were determined under controlled conditions as follows. The RIC ^{111}In-CHX-A -DTPA-Herceptin®, at a molar excess of 10:1, was incubated in a water bath in a 50 mM sodium acetate buffer and in human plasma (pH 7.4) at room temperature and 37°C for 24, 48, and 96 h postlabeling. The same procedure was used to determine the stability of ^{111}In-labeled *anti-HER2/neu* and murine IgG protein. The RIC ^{90}Y-CHX-A -DTPA-Herceptin® at a molar excess of 10:1 was incubated in a water bath at room temperature and at 37 . . . C in a PBS and in human plasma at a pH of 7.4 for 24, 48, and 96 h postlabeling. Stability of each RIC was determined using instant thin layer chromatography (ITLC) as described earlier.

Binding of ^{111}In-RICs to Human Breast Cancer Cell Lines

The binding capacity of the new ^{111}In-RICs to defined human cancer cell lines MCF-7 and SKBR-3 was tested. MCF-7 cells were cultivated in Eagle's minimum essential medium with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate, and supplemented with 0.01 mg/mL bovine insulin and 10% fetal bovine serum. SKBR-3 cells were harvested by trypsinization, washed, counted. 2.5×10^5 cells were then seeded in 12-well plates (6.25×10^4 cells/cm^2). The cells were incubated at 37¡C for 18 h and then the media was replaced with 2 mL of fresh McCoy's Medium with 10% fetal bovine serum containing one of the three RICs (1 μCi/mL). The cells were incubated in 5% CO_2 for 3 h at 37¡C. **◄ QU2** After the incubation period, the cells were washed three times with 2 mL of PBS and then solubilized with 1 mL of 1% SDS and the radioactivity bound to the cells counted in a gamma well counter.

Table 2. Specific Activity, Purity, and Efficiency of Labeling of Herceptin® with ^{111}In via CHX-A″-DTPA Chelator

	PREPARATION A*	PREPARATION B*
Specific activity: μCi ^{111}In/μg Herceptin®	1.87	3.10
Radiochemical purity (% [111]In in RIC**)	96.8	92.7
Efficiency of labeling: % [111]In bound to RIC	31.8	19.4

*Molar excess of CHX-A″-DTPA: Herceptin® was: PREPARATION A = 10:1, PREPARATION B = 50:1.
**Unreacted chelate removed by dialysis.

Table 3. Stability of Herceptin®-CHX-A″-DTPA-^{111}In in Sodium Acetate Buffer and Human Plasma

| Time (h) | Temperature | % RIC stability in: | |
		Buffer	Human plasma
0	RT	97.9	—
24	RT	97.9	95.9
	37°C	98.1	95.5
48	RT	97.8	89.6
	37°C	97.7	89.3
96	RT	95.7	90.0
	37°C	94.8	85.0

Binding of ^{90}Y-RICs to Breast Cancer Cells

The binding of ^{90}Y-DTPA Herceptin® and ^{90}Y-DTPA-*anti HER2/neu* antibody to human cancer cells MCF-7 and BT-474 was determined using various concentrations of RIC. MCF-7 cells, which express normal amounts of *HER2/neu* receptor, and BT-474 cells, which overexpress *HER2/neu* receptor, were used. Cells were exposed to three different concentrations of ^{90}Y-RICs for 24 h at 37¡C. The specific activity (μCi/μg immunoconjugate) was 1.3 for ^{90}Y-DTPA Herceptin® and 1.2 for the ^{90}Y-DTPA *anti-HER2/neu* antibody. The radiochemical purity of the ^{90}Y-DTPA Herceptin® complex was 96.6% and 94.2% for the ^{90}Y-DTPA-*anti HER2/neu* antibody complex.

RESULTS

Conjugation of Immunoglobulins

The average number of bifunctional CHX-A-DTPA molecules bound to the Herceptin® molecule, determined using the method of Pippin et al.,[11] was found to be 1.4:1. This determination was not made for p-SCN-Bz-DOTA antibody conjugates.

Labeling Immunoglobulins with [111]In Using a DOTA Linker

The results of this labeling experiment are presented in Table 1. All three antibody conjugates ◄ T1 were successfully labeled with [111]In. The RICs exceeded 89% in purity. The efficiency of labeling was calculated from the total initial radioactivity used in labeling and the radioactivity recovered in the RIC after isolation by the PD-10 column. The specific activity was calculated from the radioactivity present in the RIC isolated by the PD-10 column and the amount of IgG present in the conjugate used in the preparation of RIC. The purity of the RIC, prepared using a PD-10 column, was determined by ITLC. The radioactivity representing free radioisotope was separated from the RIC and the amount of both measured. Total radioactivity (free + RIC) was equal to 100%.

Our original intent was to use purified Herceptin® (to be obtained directly from the manufacturer) for labeling purposes. However, the manufacturer stopped supplying all purified preparations of Herceptin® for investigational purposes. Only pharmacy grade Herceptin® designed for direct infusion into patients could be obtained. Although we were able to label pharmacy grade Herceptin® with ^{111}In using the p-SCN-Bz-DOTA chelator, the immunological activity of the antibody was lost. This was determined by demonstrating the lack of binding of Herceptin®-p-SCN-Bz-DOTA-^{111}In to MCF-7 and SKBR-3 human breast cancer cells *in vitro* (data not included). For this reason, no further DOTA conjugations and labelings were performed.

Table 4. Binding of [111]In Labeled RICs to MCF-7 and SKBR-3 Human Breast Cancer Cells in 3 h at 37°C

RIC	SKBR-3	MCF-7	Binding ratio
^{111}In-CHX-A″-DTPA-Herceptin®	24,580 ± 1,405*	620 ± 18	40:1
^{111}In-CHX-A″-DTPA-HER2/neu**	33,350 ± 2,800	1,540 ± 48	22:1
^{111}In-CHX-A″-DTPA-IgG	290 ± 25	250 ± 71	1:1

*Radioactivity is expressed as average CPM per 100,000 cells ± standarad deviation obtained from three separate studies.
**Murine Antibody (Becton Dickinson, San Jose, CA) that reacts to cells overexpressing *HER2/neu*.

Table 5. Comparative Binding of ^{111}In-RIC to MCF-7, SKBR-3, HCC-1954 and BT-474 Human Breast Cancer Cells at 3 h at 37°C*

RIC	MCF-7	SKBR-3	HCC-1954	BT-474
^{111}In-CHX-A″-DTPA-Herceptin®	161 ± 14	6,310 ± 206	8,239 ± 598	4,920 ± 192
^{111}In-CHX-A″-DTPA-anti-HER2/neu	113 ± 53	2,206 ± 264	5,054 ± 336	2,875 ± 449
^{111}In-CHX-A″-DTPA-IgG	50 ± 14	50 ± 8	50 ± 5	34 ± 5

*Activity expressed as average CPM per 100,000 cells ± SD from three separate studies.

Labeling Pharmacy Grade Herceptin® with ^{111}In Using a DTPA Linker

Pharmacy grade Herceptin® was conjugated with the bifunctional metal ion chelating agent (CHX-A -DTPA) as described earlier. The yield, purity, and specific activity of the ^{111}In-labeled Herceptin® were measured for the two preparations of Herceptin® immunoconjugate. The results are presented in Table 2. The efficiency of labeling as well as the radiochemical purity was higher for the first preparation (Preparation A). However, Preparation A yielded lower specific activity than Preparation B.

The data clearly show the labeling of Herceptin® with ^{111}In using CHX-A -DTPA in our laboratory. Because of the greater radiochemical purity and efficiency of labeling obtained with the 10:1 molar excess of CHX-A -DTPA: Herceptin®, Preparation A was used in the remainder of our studies.

Stability of ^{111}In-RIC

The *in vitro* stability of Herceptin®-CHX-A -DTPA-^{111}In in sodium acetate buffer (pH 6.3) and human plasma (pH 7.4) was tested. The preparation was stable in both solutions and at both temperatures (Table 3). However, after 96 h of incubation the RIC appeared to be slightly less stable in plasma than in the acetate buffer at both temperatures.

Binding of ^{111}In-RICs to Human Breast Cancer Cells

^{111}In-RICs demonstrated binding to normal expressing HER2/neu cells (MCF-7) and to HER2/neu overexpressing cells (SKBR-3). However, ^{111}In-CHX-A -DTPA-IgG did not show significant binding to either tumor cell line. Table 4 shows the amount of radioactivity [expressed in counts per minute (CPM) per 100,000 cells] bound to both types of cells.

The RIC ^{111}-In-CHX-A -DTPA-Herceptin® binds 40 times greater to human breast cancer cells that overexpress *HER2/neu* (SKBR-3 cells) compared to normal *HER2/neu* expressing cells (MCF-7 cells). The RIC ^{111}In-CHX-A -DTPA-anti-HER2/neu binds 22 times greater to the same *HER2/neu* overexpressing cells compared to the normal *HER2/neu* expressing (MCF-7) cells. However, there is no difference in binding of the nonspecific mouse ^{111}In-CHX-A -DTPA-IgG in overexpressing and normal expressing breast cancer cells *in vitro*.

Additional binding studies with labeled Herceptin® [^{111}In-CHX-A -DTPA-Herceptin®], labeled *anti-HER2/neu* MoAb [^{111}In-CHX-A -DTPA-*anti-HER2/neu*], and labeled nonspecific IgG [^{111}In-CHX-A -DTPA-IgG] were extended to two new cell lines. The new cell lines included HCC-1954 and BT-474, both of which overexpress *HER2/neu* receptor proteins on their cell surface. The same experimental conditions described above were used and the data is summarized in Table 5.

In vitro binding studies using four separate cell lines and ^{111}In-RICs were successful. The binding of ^{111}-In-CHX-A -DTPA-Herceptin® to human breast cancer cells that overexpress *HER2/neu* (SKBR-3, HCC-1954, and BT-474 cells) is significantly higher than to normal *HER2/neu* expressing cells (MCF-7 cells). The RIC ^{111}In-CHX-A -DTPA-*anti HER2/neu* also binds greater to the same *HER2/neu* overexpressing cell lines compared to the normal

Table 6. Specific Activity, Radiochemical Purity, and Efficiency of Labeling Herceptin®-DTPA-^{90}Y

Specific Activity (μCi^{90}Y/μg Herceptin®)	2.71
Radiochemical purity (% ^{90}Y in RIC)	99.3
Efficiency of labeling (% ^{90}Y bound to RIC)	27.1

Table 7. Stability of ^{90}Y-DTPA Herceptin®

Time: hours & temperature		% RIC* stability in PBS buffer	Human plasma
24	RT	97.7	97.6
	37°C	97.0	95.4
48	RT	97.0	93.1
	37°C	88.9	87.2
96	4°C	97.1	—
	RT	95.6	86.3
	37°C	81.5	80.5

*Specific Activity of RIC = 2.71 μCi/μg.

Table 9. Binding of ^{90}Y labeled *anti-HER2/neu* to MCF-7 and BT-474 Cells for 3 h at 37°C

Cell line	Binding affinity*
MCF-7	2.46 ± 0.31
BT-474	24.37 ± 1.42

*Activity expressed as average CPM per 100,000 cells ± ±SD from three separate studies.

HER2/neu expressing cells. However, there is no difference in the *in vitro* binding of the nonspecific RIC Mouse ^{111}In-CHX-A -DTPA-IgG to the overexpressing and normal expressing breast cancer cell lines.

Labeling Pharmacy Grade Herceptin® with ^{90}Y

The labeling results of pharmacy grade Herceptin® with ^{90}Y are presented in Table 6. CHX-A -DTPA-Herceptin® was successfully labeled with ^{99}Y with a specific activity of 2.71 and an efficiency of 27%. The radiochemical purity of the RIC was found to be > 99%.

Stability of ^{90}Y-DTPA Herceptin®

The ^{90}Y- CHX-A -DTPA-Herceptin® preparation was stable in both phosphate buffered saline solution at pH 7.4 and human serum at both room temperature and 37°C (Table 7). Only in human plasma did the stability decrease slightly after 96 h.

Binding Capacity of ^{90}Y-DTPA Herceptin® and *anti-HER2/neu* Antibody

The binding results are presented in Table 8. Because ^{90}Y is a β emitter and can reduce cell re-

ceptor binding, the effect of increasing ^{90}Y concentration in the RIC was investigated. *In vitro* binding of ^{90}Y-DTPA Herceptin® and *anti-HER2/neu* antibody to BT-474 (*HER2/neu* overexpressing) cells was measured over a 24-h period. There was a linear increase in the binding of ^{90}Y-DTPA-*HER2/neu* as ^{90}Y-RIC concentration increased. This did not occur with ^{90}Y-DTPA-Herceptin® RIC suggesting that the Herceptin® is more sensitive to ^{90}Y activity than *anti-HER2/neu* antibody.

Because ^{90}Y labeled *anti-HER2/neu* showed greater binding capacity and stability, the *in vitro* binding of this RIC was tested in MCF-7 and BT-474 cells. The results are presented in Table 9.

These data demonstrate that ^{90}Y labeled *anti-HER2/neu* RIC binds 10 times more avidly to BT-474 cells (*HER2/neu* overexpressing cells) than to MCF-7 cells (normal expressing cells) *in vitro*.

DISCUSSION

The *anti-HER2/neu* antibody and nonspecific mouse IgG are preparations of protein without an excess of preservatives added and were successfully labeled with ^{111}In using a DOTA linker (Table 1) without losing their *in vitro* binding capacity to HER2/neu overexpressing cancer cells. This was not true for pharmacy grade Herceptin®.

Table 8. Binding of ^{90}Y labeled Herceptin® and *Anti-HER2/neu* to BT-474 Cells for 24 h at 37°C

μCi of ^{90}Y/μg RIC	^{90}Y-DTPA-Herceptin®	^{90}Y-DTPA HER2/neu
0.2	2.16 ± 0.40*	2.39 ± 0.35
0.4	2.48 ± 0.32	4.08 ± 0.68
0.8	2.88 ± 0.15	6.83 ± 1.89

*Activity expressed as average CPM per 100,000 cells ± SD from three separate studies.

In our hands ^{111}In-labeled pharmacy grade Herceptin® using the p-SCN-Bz-DOTA linker did not bind with human cancer cells *in vitro*. Experiments were conducted to extract Herceptin® from the pharmacy grade preparation using ammonium sulfate precipitation techniques and Protein A column filtration. After extracting Herceptin® using the above mentioned procedures, we were able to label this antibody with ^{111}In using the DOTA chelator. However, the new RIC failed to bind with tumor cells *in vitro*, suggesting the process of extraction compromised the immunoreactivity of Herceptin®. For this reason, further labeling experiments with Herceptin® were performed using the CHX-A -DTPA chelator.

As illustrated in Tables 2 and 3, pharmacy grade Herceptin®, *anti-HER2/neu* antibody, and murine IgG protein were labeled with ^{111}In using the CHX-A -DTPA chelator. These labeled complexes were produced with reasonable specific activity and high radiochemical purity. The RICs were stable over time and retained their ability to bind to receptors on several cancer cell lines, especially to those that overexpress *HER2/neu* receptors (BT-474, SKBR-3, and HCC-1954) (see Tables 3 and 4).

As illustrated in Tables 6–9, we were able to label the above mentioned antibody conjugates with ^{90}Y resulting in RICs with reasonable specific activity and high radiochemical purity. The ^{90}Y-labeled pharmacy grade Herceptin® is stable over time (Table 8). The ability of ^{90}Y-labeled pharmacy grade Herceptin® to bind to receptors on tumor cell lines, especially those overexpressing *HER2/neu* (BT-474), over 3 h has been demonstrated (Table 9).

CONCLUSIONS

Pharmacy grade Herceptin®, *anti-HER2/neu* antibody, and murine nonspecific IgG protein can be labeled with ^{111}In and ^{90}Y using the CHX-A -DTPA chelator and these labeled complexes retain their binding capacity (immunoreactivity) and are stable over time under laboratory conditions. To our knowledge these observations have not been previously published. The [111]In- and ^{90}Y-labeled Herceptin® RICs bind to breast tumor cells *in vitro*. There is significantly higher binding to breast tumor cells that overexpress *HER2/neu* receptors than to those tumor cells which express normal concentrations of *HER2/neu* receptor. This is also true for *anti-HER2/neu* antibody but not for murine nonspecific IgG protein.

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